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It has recently been reported that disruption of the circadian clock leads to increased breast cancer risk in humans and to a high rate of ionizing radiation (IR)-induced tumors in mice. Cryptochrome 1 and 2 proteins are core components of the mammalian circadian clock and mice mutated in both genes are arrhythmic. We tested *Cry1-/- Cry2-/-* mice and fibroblasts derived from these mice for radiation induced cancer and killing, and DNA damage checkpoint and killing, respectively. We find that the arrhythmic mice and fibroblasts are indistinguishable from wild-type controls with respect to their radiation-induced morbidity and mortality. We also find that upregulation of the cell cycle kinase Wee1 in the absence of Cryptochromes does not affect the DNA damage checkpoint and that c-Myc regulation is normal in the absence of Cryptochromes. Our data suggest that the disruption of the circadian clock per se does not predispose mice to cancer. It appears that the core clock proteins may participate in DNA damage checkpoints in manners unique to each core clock protein and as a consequence circadian disruption may or may not predispose mice to cancer depending on the specific mechanism of disruption.

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INTRODUCTION

The circadian clock and the cell cycle are two global oscillatory systems in most eukaryotes which are responsible for regulation of many essential pathways and processes. It has long been suspected that these two systems, both of which oscillate with a periodicity of approximately 24 hours, are somehow connected but the nature of this relationship has not been well-defined. Reports of epidemiological studies that circadian rhythm disruption due to shift work increases breast cancer risk in women (Hansen, 2001) indicate that there is indeed a link between the cell cycle and the circadian clock. More recent reports show that genetic or surgical disruption of the clock can cause increased rates of tumor growth in mice (Fu *et al*, 2002; Filipski *et al*, 2002). The goal of this work is to investigate the effect of genetic circadian clock disruption on tumorigenesis and the cell cycle in a mouse system using mice deficient in *Cryptochrome1* and *Cryptochrome2*, two of the core mammalian circadian clock genes.

The specific aims of this proposal are as follows:

1. **We will determine the effect of circadian clock disruption on uninduced tumor development in cryptochrome-deficient mice.**

2. **We will determine the susceptibility of mice with disrupted circadian rhythms to ionizing radiation.**

3. **We will use cell-based assays to investigate the cell cycle phenotype of cryptochrome-deficient cells under normal and induced conditions.**

Specific Aim 1: We will determine the effect of circadian clock disruption on uninduced tumor development in cryptochrome-deficient mice.

We have set aside a small population (11 of each genotype) of wild-type mice and *Cryptochrome1*$^{-/-}$*Cryptochrome2*$^{-/-}$ mice (*Cry1*$^{-/-}$*Cry2*$^{-/-}$, Selby *et al*, 2000; Vitaterna *et al*, 1999) for observation of tumor development under normal, uninduced conditions. Due to the inefficient breeding we observe for *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, the cohort of mice used for this study was relatively small. We are continuing to breed mice for use in this study and are observing the current cohort for mortality and tumor development.

Specific Aim 2: We will determine the susceptibility of mice with disrupted circadian rhythms to ionizing radiation.

We have set aside a population of wild-type mice (24) and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice (27) for observation of susceptibility to ionizing radiation (IR). Mice were treated with a single dose of 4 Gy of IR at approximately 8 weeks of age. Treatment was done at zeitgeber time (ZT) 10, which has been reported to be the time of day at which the mitotic index in murine bone marrow is highest (Bjarnason and Jordan, 2000). Mice were observed for 80+ weeks after treatment for morbidity and mortality and the results were analyzed using the Kaplan-Meier method of determining survival rate (Figure 1). Our results show that wild-type mice and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice show similar survival rates after treatment with IR. Moreover, no overt tumors were observed in mice of either genotype over the course of the study or as causes of death. Irradiated mice died from a variety of causes including paralysis, seizures, and genitourinary prolapses and infection that necessitated euthanasia, and in some cases indeterminable causes.

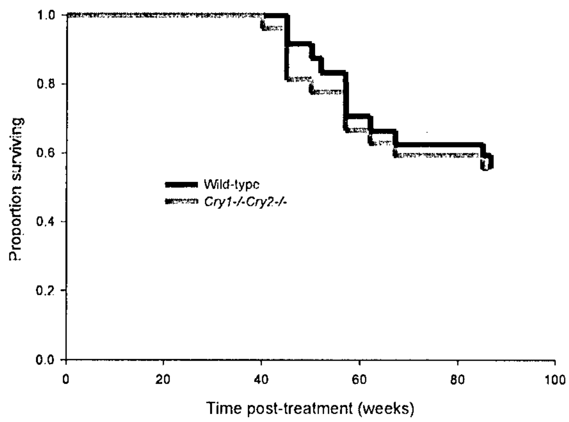

Figure 1: Crytpochrome-deficient mice do not show increased sensitivity to ionizing radiation. Mice irradiated at 8 weeks of age with a single dose of 4 Gy of IR were observed over 80+ weeks for morbidity and mortality. Survival is plotted using the Kaplan-Meier method.

(Note: Originally we intended to repeat this analysis using mice in a retinal degeneration background to confirm that any change in IR response that we observed was indeed due to the circadian phenotype of the animals. However, because our data indicates no change in IR response between wild-type and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, we elected not to perform the analysis in an alternative background.)

Specific Aim 3: We will use cell-based assays to investigate the cell cycle phenotype of cryptochrome-deficient cells under normal and induced conditions.

For this set of experiments we have used wild-type and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ fibroblast cell lines isolated from our animals (Thompson et al, 2004). Both cell lines underwent spontaneous immortalization. Our goal was to determine the cell cycle phenotype of both cell lines with respect to cell cycle arrest after DNA damage and DNA repair after damage.

We used flow cytometry to determine cellular response to IR. Cells were treated with 8 Gy of IR and DNA content analysis was performed at the indicated timepoints (Table 1). Percentage of cells in G1, S, and G2/M phases of the cell cycle was quantitated and is shown in Table 1. Our data shows that both wild-type and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ fibroblasts underwent a G2/M arrest as a result of DNA damage by IR. Moreover, both cell types recovered from the G2/M arrest with similar kinetics.

Genotype	Time (h) post-IR (8 Gy)	% cell population		
		G1	S	G2/M
Wild-type	0	45.16	30.28	**25.98**
	4	31.53	36.24	34.08
	8	14.25	31.41	**56.06**
	12	18.09	16.85	65.72
	24	39.71	27.95	**33.68**
Cry1$^{-/-}$*Cry2*$^{-/-}$	0	41.20	34.34	**26.35**
	4	24.17	42.43	36.37
	8	10.64	36.63	**56.50**
	12	21.04	9.91	68.05
	24	39.08	26.88	**32.94**

Table 1: Cellular arrest in response to IR. Cells of the indicated genotypes were treated with 8 Gy of IR and DNA content analysis was performed at the indicated timepoints.

To analyze DNA repair after treatment with DNA damaging agents, clonogenic assays were performed. Cells were plated at low density and treated with the indicated doses of either ultraviolet light (UV) or IR. After growing for 9-10 days, colonies were stained and counted. As Figures 3A and 3B show, both wild-type and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ fibroblasts show identical survival profiles after treatment with either UV or IR. These data indicate that the DNA damage checkpoint and DNA repair pathways are operating normally in both cell types and that *Cry1*$^{-/-}$*Cry2*$^{-/-}$ fibroblasts are not deficient in either of these pathways.

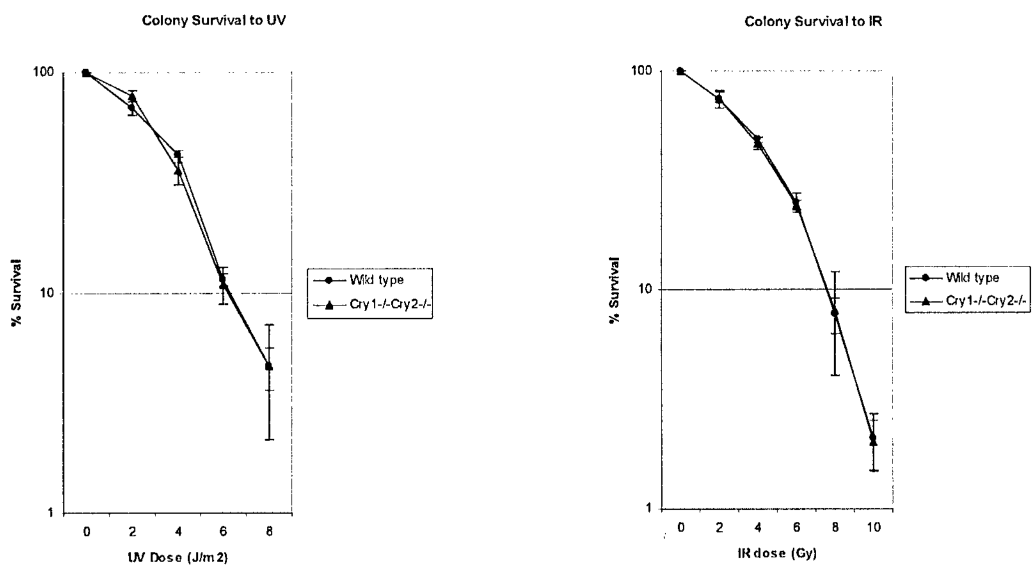

Figure 3: Cellular survival in response to UV or IR. (A) Colony survival after treatment with UV. (B) Colony survival after treatment with IR. Cells were treated with the indicated doses of either UV or IR and allowed to form colonies for 9-10 days. Colonies were fixed and stained with Giemsa stained and survival is plotted.

Future plans for this set of experiments include analysis of protein expression profiles for a panel of proteins involved in cell cycle regulation.

KEY RESEARCH ACCOMPLISHMENTS

- Mice with genetic disruption of the circadian clock through loss of core circadian clock genes *Cryptochrome1* and *Cryptochrome2* do not show increased sensitivity to IR or increased susceptibility to IR-induced tumorigenesis. **(Specific Aim 2)**
- Both wild-type and Cryptochrome-deficient fibroblasts show similar responses to DNA damaging agents with respect to DNA damage checkpoint-mediated cell cycle arrest. **(Specific Aim 3)**
- Both wild-type and Cryptochrome-deficient fibroblasts show normal abilities to repair DNA in response to DNA damage by either UV or IR. **(Specific Aim 3)**

REPORTABLE OUTCOMES

- MANUSCRIPT IN PROGRESS
  Gauger MA and Sancar A. Cryptochromeless mice lacking circadian rhythm do not exhibit increased incidence of ionizing radiation-induced morbidity or mortality. 2005 (*in progress*).

- ABSTRACT
  Gauger MA and Sancar A. Cryptochrome, circadian clock, cell cycle, and cancer. *Keystone Symposia 2005 Cancer and Development Abstract Book* 2005: 121.

- PRESENTATION
  Gauger MA. Cryptochrome, circadian clock, cell cycle, and cancer. Keystone Symposium 2005: Cancer and Development. February 5-February 10, 2005, Banff, Alberta, Canada.

CONCLUSIONS

From our current progress on the work outlined in our proposal, we conclude that circadian clock disruption per se does not predispose mice to cancer. Although it has been shown that disruption of one core circadian gene (*mPeriod2*) does cause increased tumorigenesis and disrupted cell cycle in mice, this phenomenon is not seen after disruption of the two *Cryptochrome* core circadian genes and thus is not universal to all types of circadian clock disruption. Further work is needed to discover the exact mechanism by which some types of circadian clock disruption can predispose individuals to tumor development.

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